Effect of carpropamid on secondary infection by rice blast fungus

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Abstract: Carpropamid (WIN®, KTU 3616) provides good control of leaf and panicle blast by 'one-shot' nursery-box treatment. It inhibits melanin biosynthesis in appressorial cells of *Pyricularia oryzae*, making them hyaline. Penetration by infection hyphae from the hyaline appressoria into rice epidermal cells is substantially hindered. In addition, the spread of rice blast spores from primary lesions to the other parts of the plant leading to secondary infection is largely prevented when the plants are treated with carpropamid by spray or water surface application. Secondary infection was simulated in a glass chamber fitted with an ultrasonic humidifier. On treated plants, many blast spores formed in the lesions, but the number of air spora that were dispersed from the lesions decreased significantly. A similar suppression of the spore liberation was observed *in vitro* when lesions on rice leaf segments, or discs from *Pyricularia* cultures on oatmeal agar were treated with the chemical. Spores from treated lesions or from the cultures on oatmeal agar amended with the chemical germinated normally and produced well-melanized appressoria on cellophane membranes. In addition, the spores proved to be fully pathogenic towards rice seedlings, producing normal disease symptoms. These results strongly suggest that carpropamid reduces the secondary infection of rice by *Pyricularia* by specifically hindering spore liberation.

Keywords: carpropamid; melanin-biosynthesis inhibitor; secondary infection; spore-liberation inhibition

1 INTRODUCTION

Carpropamid {(1*R*/*S*, 3*S*/*R*)-2,2-dichloro-*N*-[1-(4-chlorophenyl)ethyl]-1-ethyl-3-methylcyclopropanecarboxamide} is a chemical with high systemic activity against rice blast disease. Although mycelial growth, spore germination and appressorium formation of the causal pathogen *Pyricularia oryzae* Car are not affected, the chemical strongly inhibits melanin biosynthesis of the pathogen. Blocking melanization in appressorial cells decreases the ability of the fungus to penetrate into rice epidermal cells.[1] This effect on blast fungus suggests that carpropamid is a melanin biosynthesis inhibitor (MBI), like tricyclazole, pyroquilon or fthalide (4,5,6,7-tetrachlorophthalide). However, the target sites of carpropamid in the melanin biosynthesis pathway differ from those of other MBIs; carpropamid blocks the dehydration reactions from scytalone to 1,3,8-trihydroxynaphthalene and from vermelone to 1,8-dihydroxynaphthalene,[2,3] whereas other MBIs inhibit reduction steps in the melanin biosynthesis pathway.[4] The rigidity of the melanized layer formed outside the cell membrane is thought to be essential for maintaining high turgor pressure in the appressoria, leading to efficient penetration by the pathogen through the epidermis of the host plants.[5,6] Furthermore, blocking melanin biosynthesis also results in an accumulation of the cytotoxic intermediates.[7]

A recent study suggested that fungal melanin counteracts the rise in internal pressure caused by the osmotic gradient in appressoria, in which turgor pressure was estimated to reach as much as 8 MPa.[8–10]

As well as interfering with fungal melanin biosynthesis, MBIs seem to affect various physiological reactions of the rice blast fungus. Tricyclazole is known to influence secondary infection by conidia spread from blast lesions to other parts of the plant. The reduction in secondary infection has been ascribed to decreased spore formation in blast lesions and also to the lower virulence of the spores formed.[11] A similar effect has been described for pentachlorobenzylalcohol (PCBA) under nursery-bed conditions.[12]

Many fungicides appear to have subtle physiological effects on pathogens and host plants in addition

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to their primary actions. The observed level of control, therefore, could reflect a complex of effects on the pathogen and host plants.

Carpropamid shows only weak inhibitory activity against mycelial growth, spore formation, spore germination and appressorium formation by the blast fungus, and this may not contribute much to blast control. However, further physiological effects of carpropamid were found besides its main effect on melanin biosynthesis. Most important among these was the inhibition of the secondary infection, which cannot be detected in the usual fungicide tests. In this study the influence of carpropamid on spore formation, spore liberation and the virulence of spores was examined to investigate how the secondary infection was suppressed by the chemical.

2 MATERIALS AND METHODS

2.1 Chemicals, rice plants, blast fungus and culture method

Carpropamid was provided by Bayer AG, Leverkusen, Germany. Tricyclazole and pyroquilon were synthesised by the Chemical Research Department at the Yuki Research Centre. Fthalide 500 g kg^{-1} WP (Rabcide®, Kureha Chemical Industry Co Ltd 1-9-11 Horidome-cho, Nihonbashi, Chuo-ku, Tokyo) was purchased from the market. The chemicals were dissolved in methanol and diluted with distilled water containing 'Tween' 20 except fthalide WP, which was directly suspended in distilled water. Methanol and 'Tween' 20 were less than 1 g $litre^{-1}$ and 3 g $litre^{-1}$, respectively, in the final treatment.

Rice (*Oryza sativa* L, cv Kusabue) was used throughout the experiments. Tillering rice plants grown in Wagner pots were first inoculated with 2×10^5 spores ml^{-1} of *Pyricularia oryzae* (perfect stage: *Magnaporthe grisea*) to serve as the inoculum source for secondary infection. The extent of secondary infection was tested using young rice seedlings at the three- to five-leaf stage grown in 7-cm vinyl pots. Alluvial soil from a paddy field and artificial soil for the nursery box (Kumiai baido) were used for Wagner pots (area 100 cm^2) under irrigated conditions, and for the vinyl pots, respectively. Rice plants were grown in the greenhouse at 20 to 33°C.

P oryzae strains TH 67-22 (kindly provided by Dr Yaegashi, Saga University, Saga City, Japan) and P_2 were used in the experiments. These strains were maintained on PDA slants and cultured for experimental work on oatmeal agar plates kept under BLB lamp (black light blue, 290–410 nm) to induce sporulation.

2.2 Secondary infection and trapping of dispersed spores in the chamber

To stimulate the incidence of disease spread from leaf blast lesions, glass chambers (0.7 m deep × 0.7 m high × 2.5 m wide) fitted with an ultrasonic humidifier (National FE-05KYJ) were installed in the greenhouse. The temperature in the chamber was maintained at 24–26°C and the humidifier was run for 4 × 15 min per night.

Rice plants at the tillering stage grown in Wagner pots were inoculated with a spore suspension of the strain TH 67-22. Nine days after inoculation, carpropamid (0–150 mg $litre^{-1}$) was sprayed on the infected rice plants. Three of these pots were used as infection source in the following studies.

For the systemic tests, 10 ml of carpropamid solution at 400 μg ml^{-1} were pipetted into irrigation water of the Wagner pot (area 100 cm^2) of rice plants at the tillering stage at a rate equivalent to 4 kg AI ha^{-1}. Four days after treatment the rice plants were inoculated with a spore suspension of strain TH 67-22. Obviously, the time interval of four days was too short for carpropamid to provide a sufficient control of the disease on the rice leaves; thus numerous blast lesions developed on the leaves. Nine days after inoculation three pots with well-developed lesions were used as infection source in the glass chamber. To study the effect on secondary infection, several pots of healthy rice seedlings at the three- to four-leaf stage were placed in the glass chambers along with infected rice plants for two nights. Symptoms developing from the primary lesions as a result of secondary infection were assessed after 10 days' incubation in the chamber.

Air spora dispersed from the source plants were trapped on glass slides coated with glycerine jelly. For this purpose, the glass slides remained for seven days in the glass chamber together with the inoculum sources, and the numbers of spores trapped on the surface of the glass slide were counted.

2.3 Leaf-segment method

The leaf-segment method was used to test the effect of the chemicals on spore formation and spore liberation from the treated blast leaf lesions. Infected rice plants were prepared as described above. Before spray treatment, the blast lesions were washed with tap water using a paint brush to remove the blast spores that had already been formed.

Leaf segments (approx. 1.5 cm) with a blast lesion were cut from diseased rice leaves one day after spray treatment (10 days after inoculation).

2.3.1 Formation, germination and pathogenicity of blast spores formed in lesions treated with carpropamid

Leaf segments sprayed with 50–200 mg carpropamid $litre^{-1}$ were placed on wet filter paper, adaxial surface upwards and kept in plastic boxes under most conditions. The plastic boxes were placed under BLB (black light blue) light for two days to enhance sporulation. Blast spores formed on 20 lesions were collected using a brush with 20 ml of distilled water containing 'Tween' 20 and the number of spores was assessed by using a binocular microscope (× 100).

After the spores had been centrifuged three times in distilled water to eliminate the chemical, they were

subjected to germination tests on cellophane membranes. The spore concentration was adjusted to 10^5 spores ml^{-1} and sprayed onto young rice seedlings to investigate their pathogenicity.

2.3.2 Spore liberation from the lesions sprayed with chemicals

To test the effect of the chemicals on spore liberation from the lesions, five leaf segments treated with the test chemicals at 2.5–10 mg $litre^{-1}$ were attached to the underside of lids of 9-cm Petri dishes of water-agar plates, which were kept under BLB light at 25°C for two to seven days. The number of spores dropping onto the surface of water agar plates was determined by microscopic methods.

2.4 Properties of blast spores cultured on oatmeal agar amended with carpropamid or other MBIs

2.4.1 Influence on spore formation, spore germination, appressorium formation and pathogenicity to rice

Strains P_2 and TH 67-22 were cultured for four days under BLB lamp irradiation on oatmeal agar plates amended with the test compounds at 2–10 μg ml^{-1}. After removal of the aerial hyphae using a plastic spatula, the cultures were kept under BLB irradiation for three days. The spores formed on the surface were collected using a paint brush in 25 ml of distilled water per plate and filtered through four layers of cheese cloth. To compare the effect of the chemicals on spore formation, the number of spores in the suspension was assessed using a hemocytometer under a binocular microscope (× 100). The spores were then centrifuged three times in distilled water (750*g*, 10 min) and used for the spore germination tests. The spore density of the suspension was adjusted to 10^5 spores ml^{-1}. Then the suspensions were inoculated onto young rice seedlings to examine their pathogenicity.

2.4.2 Blast spore liberation from the surface of oatmeal agar discs

Strains P_2 and TH 67-22 were cultured for four days on oatmeal agar plates amended with the test compounds at 2.5–10 μg ml^{-1}. After removal of the aerial hyphae, mycelial agar disks (15 mm in diameter) were cut from the culture plates. The agar discs were placed on the underside of lids of Petri dishes, mycelial side down and kept under BLB light irradiation for one night. The lids with the discs were placed over water agar. The plates were kept under BLB light at 25°C for two to seven days. The numbers of spores dropping onto the surface of the water agar were counted under a binocular microscope.

3 RESULTS

3.1 Secondary infection

Healthy rice seedlings were infected by spores dispersed from leaf blast lesions on neighbouring diseased rice plants when they were incubated together for two nights in the glass chamber fitted with an ultrasonic humidifier.

If rice blast lesions on the inoculum source were sprayed with carpropamid at 25–150 mg $litre^{-1}$, tricyclazole at 100 mg $litre^{-1}$ or fthalide at 400 mg $litre^{-1}$ before the co-incubation, the spread of blast disease to healthy rice seedlings was significantly suppressed (Fig 1A). A concentration as low as 50 mg $litre^{-1}$ of carpropamid provided satisfactory control of secondary infection. Other MBIs such as tricyclazole and fthalide reduced the rate of secondary infection to a similar extent.

To see the effect of a systemic treatment on secondary infection, the importance of the time interval between treatment and inoculation was studied, and the dosage of the chemical that still allowed spore formation on the treated plants was determined. Ten millilitres of carpropamid solution at 400 μg ml^{-1} pipetted on to a Wagner plot (area 100 cm^2) four days prior to inoculation did not prevent lesion development and allowed spore formation. However, when rice plants treated in this way were used as an infection source, secondary infection was greatly reduced (Fig 1B). If the interval was extended to more than seven days, carpropamid demonstrated its

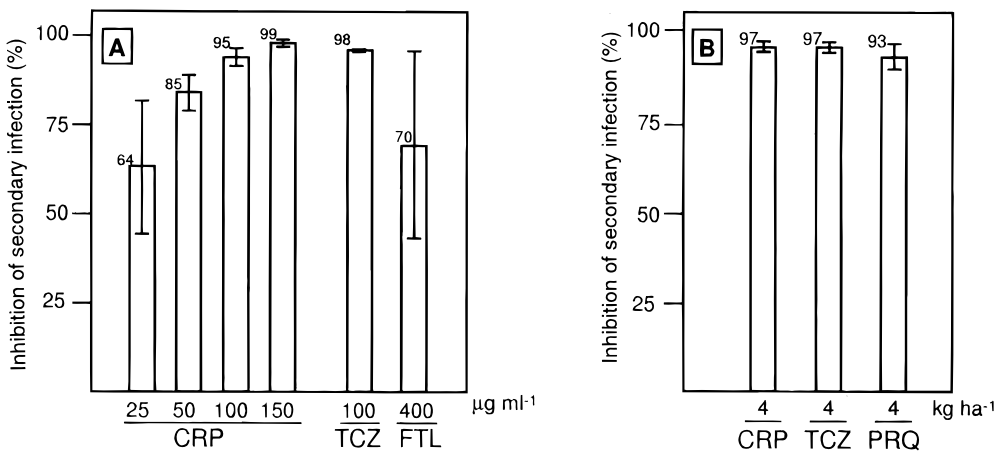

Figure 1. Inhibition of secondary infection by MBIs following (A) foliar spray or (B) submerged application. CRP: carpropamid, TCZ: tricyclazole, FTL: fthalide, PRQ: pyroquilon. Inoculation timing for water surface application: four days after treatment by CRP, one day after treatment by TCZ and 10 h after treatment by PRQ

full activity and no blast lesions were observed on the inoculated plants. However, when the interval was reduced to less than two days, secondary infection was not affected. These results suggest that it takes four to five days for the active ingredient to reach the leaf in sufficient amounts for the control of secondary infection.

In the second experiment, air spora were trapped on glass slides painted with glycerine jelly. Considerably fewer blast spores were trapped on the slide if plants were sprayed with carpropamid (Table 1). Only 3.9 and 0.4% of the spores in the control treatment were trapped on the slides when the plants were sprayed with carpropamid at 100 and 150 mg litre^{-1}, respectively.

3.2 Effect of chemicals on spore formation in blast lesions and spore pathogenicity

The decrease in the number of air spora in the chamber seemed to reflect the suppression of secondary infection observed when the infected rice plants used as an inoculum source were treated with carpropamid. In order to analyse this effect, spore formation was tested on leaf segments kept under moist conditions. The spores formed on the lesions were collected in 20 ml of distilled water from each of the 20 lesions. If carpropamid and tricyclazole at 50–200 mg litre^{-1} were sprayed onto the lesions, the number of spores decreased to 26–51% of those from the untreated lesions. Fthalide at 100–400 mg litre^{-1} did not show such a marked influence on spore formation as carpropamid and tricyclazole. Although the latter chemicals suppressed spore formation, the effect of suppression was insufficient to explain the inhibition of secondary infection by them (Table 2, Fig 1A). Therefore, factors other than spore formation seem to be responsible for the suppression of the secondary infection. In the spore germination tests, the spores formed in treated lesions germinated normally and developed normal appressoria on cellophane membranes; almost all of the appressoria formed were well pigmented (Table 1). These results indicate that MBIs do not have much influence on the germinating ability of the spores formed. Furthermore, spores obtained from blast lesions treated with carpropamid and other MBIs showed normal pathogenicity to young rice seedlings.

3.3 Spore liberation from the lesions

The numbers of air spora in the glass chamber markedly decreased when the blast lesions were treated with carpropamid, but spore formation in the lesions was not sufficiently affected to explain this phenomenon. The results obtained from the leaf-segment

Table 1. Dispersal of rice blast spores from the lesions in glass chamber

Chemicals	*Concentration* ($\mu g\ ml^{-1}$)	*Number of spores trapped*[a]	*Percentage of untreated*
Untreated	—	573 (±27.58)	
Carpropamid	50	116 (±20.96)	20.2
	100	22.3 (±4.66)	3.9
	150	2.3 (±1.23)	0.4

[a] Numbers of spores trapped on glass slides in seven days were counted. The figures indicate the average of three replicated experiments.

Table 2. Effects on MBIs on spore formation in blast lesions and on pathogenicity[a]

Chemicals	*Concentration* ($\mu g\ ml^{-1}$)	*Spore formation* (%)	*Appressoria*[b] *pigmentation*	*Pathogenicity*[c]
Untreated	—	100	+++	100
Carpropamid	50	51	+++	60
	100	34	+++	75
	200	29	+++	86
Tricyclozole	50	30	+++	74
	100	26	+++	97
	200	33	+++	95
Fthalide	100	69	+++	93
	200	71	+++	100
	400	64	+++	97

[a] Blast spores were collected from 20 lesions in 20 ml of distilled water containing 'Tween' 20. Number of spores per ml in untreated control was 42.2×10^4. Before inoculation, spores were washed three times with distilled water by centrifugation. Blast strain T 67-22 was used. Lesion area in untreated plants: 15.2%

[b] Pigmentation in appressorial cells: not pigmented = −, slightly = +, medium = ++, clearly = +++.

[c] Percentage of lesion area inoculated with the treated or untreated spores (average of two tests).

Chemicals	*Concentration* ($\mu g\,ml^{-1}$)	*Number of spores found on the water agar surface*[b] *after incubation for (days)*		
		2	*4*	*7*
Untreated	—	331.5	463	474.5 (100.0)[c]
Carpropamid	2.5	39	54	58.5 (12.3)
	5	28	52.5	52.5 (11.1)
	10	0	23	24 (5.1)
Tricyclazole	2.5	82.7	144	424.0 (89.4)
	5	14	40.3	119 (25.1)
	10	0	12	13 (2.7)
Fthalide	10	323	362	362.0 (76.3)
Pyroquilon	10	85	87	91.0 (19.2)

Table 3. Spore liberation from leaf blast lesions as determined by the leaf-segment method[a]

[a] Five leaf segments inoculated with strain TH 67-22 were incubated in the moist chamber. The numbers of spores found on the water agar were assessed on the 2nd, 4th and 7th day.
[b] Number of the dropping spores accumulated (average of five lesions).
[c] No of spores treated/No of spores untreated $\times 100$ in parentheses.

method suggest that the phenomenon is due to the reduction in spore liberation (Table 3). The number of spores found on the water agar was significantly lower if the leaf segments were treated with 2.5–10 mg carpropamid litre^{-1}. The test results demonstrated that other MBIs such as tricylazole and fthalide also affected spore liberation from blast sporophores.

3.4 Influence of carpropamid on spore formation on oatmeal agar, germination and pathogenicity

To test the activity of the test compounds on spore formation *in vitro*, spore production on oatmeal agar amended with MBIs (2–10 $\mu g\,ml^{-1}$) was evaluated. Many spores were formed on agar in the presence of MBIs; the numbers of spores fluctuated and was not clearly related to the concentration of MBIs tested.

Under normal growing conditions, the untreated spore mass of *P oryzae* appeared grey. In the presence of carpropamid and fthalide the spore mass was brown, while it was slightly pink for tricyclazole treatment. These changes in colour suggested that these MBIs were translocated to spore cells and the melanin synthesis of the latter was inhibited as in appressoria. Nevertheless, the unmelanized spores germinated normally and produced well-pigmented appressoria on cellophane membranes (data not shown). Furthermore, there was no difference in pathogenicity when melanized or unmelanized spores were used for inoculation onto young rice seedlings.

3.5 Spore liberation from *Pyricularia oryzae* cultures on oatmeal agar

Decreasing spore dispersal in glass chambers and suppressing spore liberation from blast lesions on treated leaf segments were considered to be the factors responsible for reducing the secondary infection rate (Tables 1 and 2). To confirm this, spore liberation from a *P oryzae* colony grown on oatmeal agar amended with MBIs was examined. Even at a concentration as low as 10 $\mu g\,ml^{-1}$, MBIs had a strong inhibitory effect on spore liberation from blast sporophores as shown in Table 4.

These results clearly demonstrate that decreased spore liberation from lesions, rather than a reduced spore formation or spore pathogenicity, is responsible for the suppression of secondary infection.

4 DISCUSSION

The formation, release and dispersal of spores are crucial events in the life cycle of pathogenic fungi; the prevalence of disease depends upon them. Infection of new plants by inoculum from blast lesions on infected plants (secondary infection) is commonly observed in rice fields. The number of air spora in the paddy field is closely related to the subsequent incidence of the disease. Fluctuation in the number of spores in the field could be used to forecast disease outbreaks and to anticipate the amount of damage in rice culture.[13,14]

Secondary infection by rice blast spores occurred readily in glass chambers fitted with an ultrasonic humidifier. Spore formation in blast lesions was enhanced by the high humidity. The gentle air stream produced by the humidifier circulated within

Table 4. Spore liberation from sporophores of *Pyricularia oryzae* formed on oatmeal agar culture

Chemicals	*Conc.* ($\mu g\,ml^{-1}$)	*Number of spores trapped*[a]	*Percentage of untreated*
Untreated	—	7859 ($\pm$721.80)	
Carpropamid	2.5	2365.5 ($\pm$454.55)	(30.1)
	10	204.3 ($\pm$ 99.05)	(2.6)
Tricyclazole	2.5	3379.4 ($\pm$385.70)	(43.0)
	10	1202.4 ($\pm$299.08)	(15.3)
Fthalide	2.5	3410.8 ($\pm$793.39)	(43.4)
	10	2011.9 ($\pm$652.60)	(25.6)

[a] Numbers of spores found on the surface of water agar plates were counted for a week (average of four agar discs ($\pm$SD).

the chamber and transported spores from the lesions on the inoculum source to the healthy rice seedlings. Using this inoculating system, we demonstrated that the number of air spora in the chamber was markedly reduced when the inoculum source was treated with carpropamid. Reducing the number of air spora led to the suppression of secondary infection.

The inhibition of spore formation by fungicidal chemicals has been reported. Okamoto *et al*[15] found that spore formation was inhibited by treatment with phenylmercuric acetate (PMA), and Kado *et al*[16] showed that the number of spores formed decreased after spraying iprobenfos. Sumi *et al*[12] observed that the number of air spora in nursery beds markedly decreased by spraying with PCBA (pentachlorobenzyl alcohol, an MBI). They found that PCBA strongly decreased spore formation in lesions on rice plants, but not in those on leaf segments. Tricyclazole was reported to suppress secondary infection by inhibiting spore formation in lesions and/or by causing the spores to be less virulent.[11,17]

Carpropamid, which has no direct fungicidal activity, reduced somewhat the number of spores formed in treated lesions (Table 2). However, suppression of secondary infection cannot be fully attributable to the decrease in spore production in treated lesions.

Spore release is often passive, through wind and rain, but many fungi have developed active mechanisms of spore discharge such as hygroscopic movement of sporophores and cell burst under conditions suitable for infection.[18,19] Inhibition of spore liberation from sporophores by chemicals has not been well studied, and is only rarely cited in the literature. In experiments on the effect of tricyclazole on secondary infection, Takanashi *et al*[20] observed a decrease in the dispersal of spores in moist chambers and in nursery beds. They inferred that suppression of spore liberation from blast lesions occurred.

In the present study, carpropamid markedly inhibited the liberation of blast spores from treated lesions (Table 3) and from cultures on plates of oatmeal agar amended with the chemical (Table 4). The spores formed in the lesion and on the amended medium were difficult to release from the sporophore even by intentional shock. This suggests that inhibition of spore liberation rather than a reduction in spore formation contributes to the suppression of secondary infection of rice blast.

Many fungicides have secondary action mechanisms in addition to their primary mode of action. Their effects in the field are the results of the sum of such mechanisms, although the secondary mode of action is usually not distinct. Carpropamid provides sufficient blast control through inhibition of melanin biosynthesis. In the field test with small plots, inhibition of spore liberation may not contribute to its overall control activity because the reduced inoculum in the treated plots can easily be supplemented by that deriving from surrounding areas. However, when used across a large area, or in a restricted growing area such as a small valley where the supply of inoculum is limited, the chemicals may provide a higher level of control than expected from only the inhibition of melanin biosynthesis.

There are several reports on charges in the virulence of blast spores treated with tricyclazole. Okuno *et al*[21] found that blast spores from leaf blast lesions treated with tricyclazole, or from cultures on V-8 juice agar containing tricyclazole, produced hyaline appressoria and lost their ability to penetrate cellophane membranes. They suggested that tricyclazole, after absorption into the spore cells, is transferred to the appressoria, where it inhibits melanin biosynthesis. In our study, blast spore masses formed on oatmeal agar containing carpropamid, fthalide or tricyclazole appeared brown or light pink; this indicated that these compounds affected melanin biosynthesis in spore cells. However, regardless of melanization inhibition in the spore cells, spore germination and appressorium formation on cellophane membranes were little different from controls, and the appressoria produced from the unmelanized spores were well-pigmented. Furthermore, when the unmelanized spores were inoculated on young rice seedlings, typical rice blast symptoms developed on the leaves.

These results suggest that the suppression of secondary infection by carpropamid was not due to a decrease in the virulence of treated spores, but rather to a strongly limited spore liberation from the sporophores.

Spore liberation is thought to be stimulated by diurnal rhythms of light and darkness resembling the natural alternation of day and night,[22,23] although spore formation in lesions is enhanced by high relative humidity, appropriate temperature and rain.[24] Ingold[25] observed microscopically the pedicel between blast spores and the sporophore, showing that spores are attached to their sporophores by minute stalk cells. However, detailed observation by Hashioka *et al*[26] using scanning electron microscopy indicated that the independent cell was not found in the pedicel part and that the spore hilum is attached directly to the denticle of the terminal cell of the sporophore. The dehiscence of blast spores at the denticle and the hilum seems to be promoted by swelling of the hyaloplasm due to absorption of moisture.[27] As all MBIs tested interfered with blast spore liberation to some extent, melanin accumulation in the spore may be related to the absorption of moisture and to a rise in turgor pressure by enlargement of the hyaloplasm to split them.

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